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End-point RPA-CRISPR/Cas12a-based detection of *Enterocytozoon bieneusi* nucleic acid: rapid, sensitive and specific

Yilin Wang[1,2†], Fuchang Yu[1,2,3†], Kaihui Zhang[1,2], Ke Shi[1,2,4], Yuancai Chen[1,2], Junqiang Li[1,2], Xiaoying Li[1,2] and Longxian Zhang[1,2*]

Abstract

Enterocytozoon bieneusi is a common species of microsporidia that infects humans and animals. Current methods for detecting *E. bieneusi* infections have trade-offs in sensitivity, specificity, simplicity, cost and speed and are thus unacceptable for clinical application. We tested the effectiveness of a previously reported CRISPR/Cas12a-based method (ReCTC) when used for the nucleic acid detection of *E. bieneusi*. The limit of detection (LOD) and the specificity of the expanded ReCTC were evaluated using prepared target DNA, and the accuracy of the ReCTC-based detection of *E. bieneusi* in clinical samples was validated. The ReCTC method was successfully used for the nucleic acid detection of *E. bieneusi*. The sensitivity test indicated an LOD of 3.7 copies/μl for the ReCTC-based fluorescence and lateral flow strip methods. In specificity test involving other common enteric pathogens, a fluorescent signal and/or test line appeared only when the sample was positive for *E. bieneusi*. These results demonstrated that the ReCTC method can successfully detect *E. bieneusi* in clinical samples. The ReCTC method was successfully used to detect *E. bieneusi* nucleic acid with high sensitivity and specificity. It had excellent performance in clinical DNA samples and was superior to nested polymerase chain reaction. Furthermore, the ReCTC method demonstrated its capability for use in on-site detection.

Keywords *Enterocytozoon bieneusi*, Recombinase polymerase amplification, CRISPR/Cas12a, Visualized detection, On-site detection

Introduction

Microsporidia are obligate intracellular eukaryotic parasites that infect many invertebrates and vertebrates, including humans [1, 2]. More than 17 species of microsporidia have been detected in humans with *Enterocytozoon bieneusi* being the most common [3, 4]. In addition to infecting humans, *E. bieneusi* can also infect wild animals, domestic livestock and companion animals, etc. These animals infected with *E. bieneusi* pose a significant threat to public health [5]. Microsporidiosis caused by *E. bieneusi* usually causes diarrhea and other gastrointestinal symptoms, especially in immunocompromised individuals [6–9]. *Enterocytozoon bieneusi* not only

†Yilin Wang and Fuchang Yu contributed equally to this work.

*Correspondence:
Longxian Zhang
zhanglx8999@henau.edu.cn
[1]College of Veterinary Medicine, Henan Agricultural University, Zhengzhou, Henan, P. R. China
[2]International Joint Research Center for Animal Immunology of China, Zhengzhou, Henan, P. R. China
[3]College of Animal Science, Tarim University, Alar, Xinjiang, P. R. China
[4]School of Medicine, Xinxiang University, Jinsui Road 191, Xinxiang 453003, China

threatens human health, but also affects the production performance and value of animals [3]. There is also a relationship between *E. bieneusi* infection and the etiopathogenesis of colon cancer [10]. Fumagillin is the only drug that can effectively be used to treat this disease. However, because of its high toxicity and side effects, it is not commercially available in most countries [11].

Current, the best method to detect *E. bieneusi* relies on transmission electron microscopy (TEM) to identify the polar filament and other ultrastructural characteristics [12]. However, TEM is labor-intensive and time-consuming, requires expensive equipment and specialized expertise [12]. Light microscopy-based methods are faster and typically more sensitive than TEM, but successful interpretation requires special staining methods, experienced pathologists, and the method cannot identify genus or species of *E. bieneusi* [13]. Polymerase chain reaction (PCR) is an assay based on the internal transcription spacer (ITS) of *E. bieneusi* and gel migration [14]. The PCR method is widely used because of its high sensitivity and ability to genotype the detected *E. bieneusi* isolates. However, PCR requires specialized instruments and is time-consuming (approximately 2 h for the most commonly used nested PCR). The real-time PCR method must rely on expensive real-time fluorescence quantitative PCR instrument [16]. The PCR method also involves tedious operations, which increase the risk of sample cross-contamination [12]. Therefore, these methods are unsuitable for on-site clinical disease monitoring, especially in resource-limited areas. Thus, a rapid, sensitive and specific method to detect *E. bieneusi* is needed (Fig. 1).

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) protein systems can recognize and cleave specific nucleic acid sequences (namely the *cis*-cleavage) [15]. Cas12a, a single RNA-guided endonuclease of the Class 2 Type V CRISPR/Cas system, exhibits nonspecific *trans*-cleavage activity by cleaving non-target sequences after recognizing a specific target sequence [17, 18]. This collateral effect has been developed into Cas12a-based nucleic acid detection methods and used to detect many pathogens including SARS-CoV-2 and *Enterocytozoon hepatopenaei* [19–22]. We previously integrated recombinase polymerase amplification (RPA) with the Cas12a/crRNA trans-cleavage system, termed ReCTC, establishing an

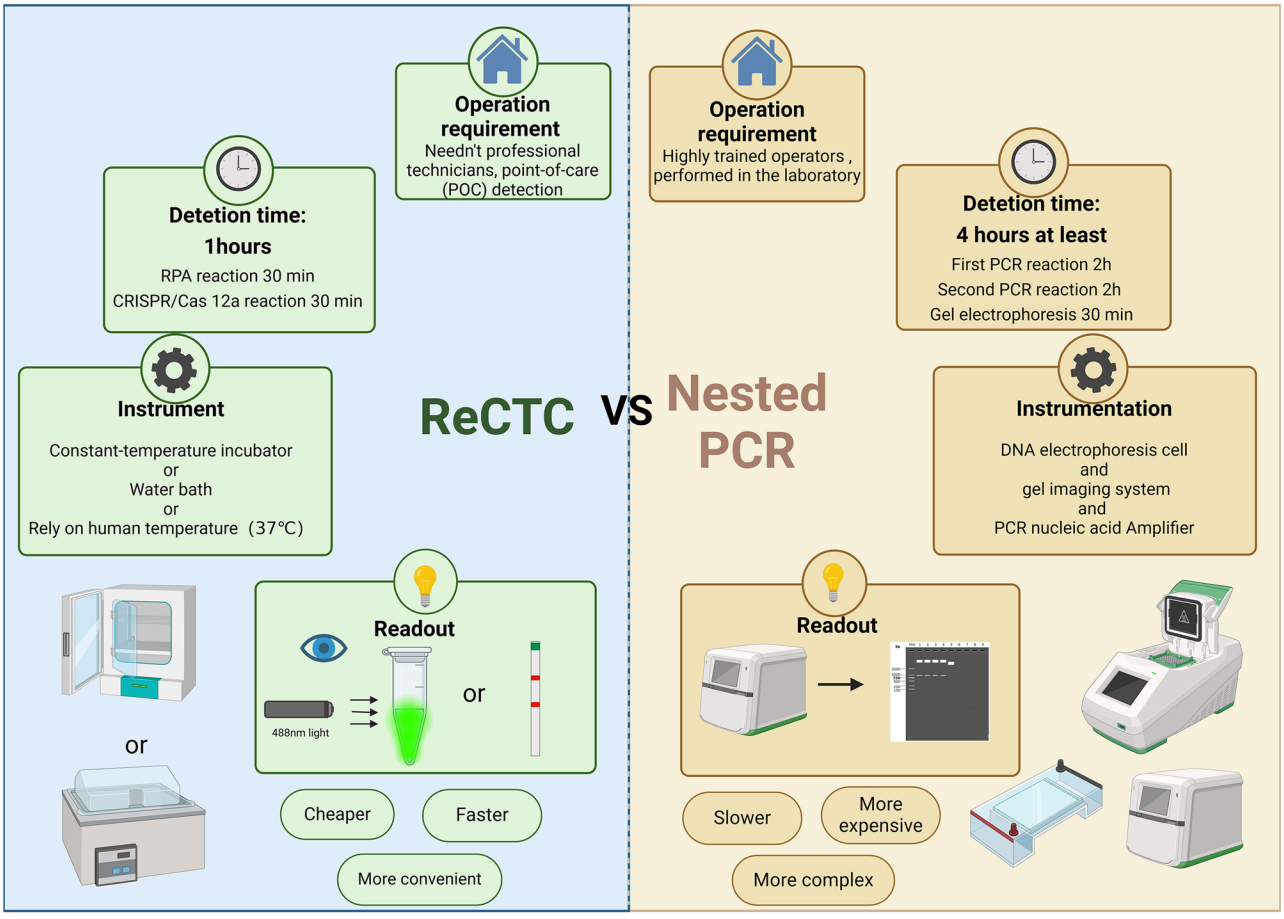

Fig. 1 Comparison of ReCTC-based detection method and nested PCR method

end-point diagnostic by observing fluorescence readouts under UV light and an on-site diagnostic using a lateral flow strip (LFS) biosensor to rapidly detect *Cryptosporidium parvum* IId subtype family nucleic acid [23]. In this study, our goal was to expand the application of the ReCTC and establish an end-point nucleic acid detection method for *E. bieneusi*.

Materials and methods

Reagents and oligonucleotides

All the primers, quenched fluorescent DNA reporter (FAM-TTATT-BHQ1) and LFS test reporter (FAM-TTATT-Biotin) were obtained from Sangon Biotech (Shanghai, China). All the nucleotide (nt) sequences are listed in Additional File 1: Table S1. Recombinant *Francisella novicida* Cas12a (FnCas12a) protein was purchased from Tolo Biotech (Shanghai, China). HiScribe™ T7 High Yield RNA Synthesis Kits were purchased from New England Biolabs (Ipswich, MA, USA). The NucAway™ Spin Columns were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The TwistAmp® Basic Kit was purchased from TwistDx Ltd. (Hertfordshire, AL, UK). The LFS biosensor was purchased from Zoonbio Biotechnology (Nanjing, China). Recombinant DNase I (RNase free) and RNase inhibitor were purchased from TaKaRa Bio Inc. (Beijing, China). Other regents used were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China).

Design and preparation of crRNA

The most commonly reported microsporidia species in humans are *Encephalitozoon intestinalis*, *Encephalitozoon. hellem*, *Encephalitozoon. cuniculi* and *E. bieneusi*. Significant heterogeneity of the spore wall protein (*swp*) gene in these species has been reported [24, 25], and we chose this gene as the target sequence for the design of crRNA. To determine the highly conserved regions with species specificity, sequences of the four species were downloaded from GenBank and aligned using ClustalX 2.1 (http://www.clustal.org/clustal2/). A 20-nt sequence closely following a T nt-rich protospacer adjacent motif (PAM) was used as the target sequence and synthesized containing the T7 promotor (Additional File 1: Table S1). To confirm the specificity of crRNA sequences used in this assay, we have performed a BLAST analysis of crRNA. We synthesized two reverse complementary crDNA oligonucleotides, and these were annealed to form dsDNA templates. The crRNA was transcribed using the HiScribe™ T7 High Yield RNA Synthesis Kit, followed by DNase I digestion and NucAway™ Spin Column purification. The concentration of purified crRNA was measured using NanoDrop One (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Recombinase polymerase amplification assay

Isothermal RPA was used to increase the concentration of target double-stranded (ds) DNA. Ten pairs of RPA primers were designed (Additional File 1: Table S1), and the TwistAmp® Basic Kit was used to conduct the RPA assay according to product instructions. Briefly, a 50-µl total reaction volume comprised 29.5 µl of rehydration buffer, 500 nM of each primer, 5 µl of extracted DNA, 2.5 µl of 280 nM magnesium acetate (MgOAc) and sterile nuclease-free water. Each reaction volume also contained the TwistAmp reaction pellet. The reaction was performed at 37 °C for 30 min. MgOAc was added to the inner surface of the tube lid while all the other reagents were added to the bottom of the tube that was then capped. Then the tube was centrifuged to mix MgOAc with the solution and trigger the RPA reactions.

FnCas12a/crRNA trans-cleavage assay

FnCas12a trans-cleavage assays were performed using a previously described method [23]. The real-time fluorescence value was recorded using a qTOWER3GqPCR system (Analytik Jena, Germany) every 30 s at a constant temperature of 37℃ (515 nm excitation wavelength and 545 nm measurement wavelength). We examined the feasibility of using the constructed LFS assay to make the test results readable to non-specialists for on-site diagnosis of *E. bieneusi*. The LFS assay was also performed using previously described methods, and the optimal concentration of reporter for the ReCTC-based LFS detection method was set at 20 nM [23].

Preparation of target DNA of *E. bieneusi* at a known concentration

The DNA fragment of the *E. bieneusi swp* gene with known concentration used in the sensitive test was prepared with PCR using the primers Ebswp-F and Ebswp-R. This was followed by gel extraction purification using SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The final concentration of the purified DNA fragment of the *E. bieneusi swp* gene was determined on NanoDrop One (Thermo Fisher Scientific Inc., Waltham, MA, USA), followed by tenfold serial dilutions.

PCR amplification of *E. bieneusi swp* gene and the ITS sequence

To compare the results of the new ReCTC assay and the nested PCR assay based on the ITS sequence for the detection of *E. bieneusi* nucleic acid, eleven known *E. bieneusi*-positive samples that were collected from hospitalized patients in Zhengzhou City and stored in our laboratory were tested using both methods [7]. PCR amplifications based on the *swp* gene and the ITS sequence were both conducted as in previous studies

with some modifications [14, 25]. Briefly, in the amplification of the *E. bieneusi swp* gene, the 20-µl total reaction volume was made up of 2 µl of 10× PCR buffer, 625 nM of each primer, 200 µM of each dNTP, 2 µl of extracted DNA, 1 unit of r*Taq* DNA polymerase (TaKaRa, Beijing, China) and sterile nuclease-free water. The reactions were conducted for 35 cycles (94 °C for 45 s, 52 °C for 45 s, and 72 °C for 60 s) with an initial denaturation at 94 °C for 5 min, followed by a final extension at 72 °C for 7 min.

The reaction system of the nested PCR based on the ITS sequence included 2 µl of DNA or first PCR product (for the secondary PCR), 1× KOD-Plus buffer, 200 µM of each dNTP, 1 mM MgSO4, 300 nM of each primer and 1.5 units of KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The PCR protocol consisted of a 5-min initial denaturation at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 57 °C (primary PCR) or 55 °C (secondary PCR) and extension for 45 s at 72 °C with a final 7-min extension step at 72 °C.

Samples information

The validation samples (11 known *E. bieneusi*-positive samples and 50 *E. bieneusi*-negative samples) and positive control used in this study were both collected from hospitalized patients in Zhengzhou City, Henan Province. The positive sample of *C. parvum* and *C. ryanae* were collected from a dairy farm in Xinxiang City, Henan Province. The positive sample of *Cyclospora cayetanesis* was collected from hospitalized patients in Kaifeng City, Henan Province. The positive sample of *Giardia duodenalis* was collected from hospitalized patient in Kafr El Sheikh Province, Egypt. The above samples are stored in our laboratory. Genomic DNA was extracted using the E.Z.N.A.TM Stool DNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions. The positive DNA solution of *E. intestinalis*, *E. hellem* and *E. cuniculi* were obtained from the institute of Sericulture and Systems Biology, Southwest University, China. Positive samples were identified by PCR amplification, sequencing and phylogenetic analysis.

Results

Design and preparation of crRNA

A fragment, conserved in *E. bieneusi* but not in the other common human microsporidia species, was identified by multiple alignment of the *swp* gene. A 20-nt sequence of this fragment was selected as the target sequence, and the crRNA sequence was also determined (Additional File 1: Table S1; Fig. S1). The BLAST result of crRNA sequence showed that, although the percent identity of crRNA sequence was 100% to other species, the sequence coverage was only 78–86% (Additional File 1: Fig. S2). By annealing synthesized single-strand crDNA oligonucleotides and in vitro transcription, crRNA was prepared. The concentration of this crRNA was measured as 1145.6 ng/µl using NanoDrop One (Additional File 1: Fig. S3 and Fig. S4).

Feasibility verification of the ReCTC assay

We expanded the ReCTC-based fluorescence and LFS methods for *E. bieneusi* detection by changing the crRNA, and the feasibility of this was verified using four known *E. bieneusi*-positive samples. The primers, named EBRPAF89 and EBRPAR334 (Additional File 1: Table S1), showed the highest amplification efficiency in the RPA assay (Additional File 1: Fig. S5) and were used in the present study. In the ReCTC-based fluorescence detection, the FAM-TTATT-BHQ1 reporter was cleaved, and an obvious fluorescence signal under UV light was visually observed, which was absent in the negative reaction (Fig. 2a). A similar distinction was also recorded using the qTOWER^{3}G qPCR system (Analytik Jena, Germany) (Fig. 2b). In the ReCTC-based LFS detection assay, a test line was seen within eight minutes on the strip when the FAM-TTATT-biotin reporter was cleaved in the positive reaction. This test line was not seen in the negative reaction (Fig. 2c).

Preparation of target DNA of *E. bieneusi* with known concentration

PCR amplification and gel extraction were used to prepare a fragment of the *E. bieneusi swp* gene. NanoDrop One analysis showed that the average final concentration of the purified DNA fragment was 22.85 ng/µl (Additional File 1: Fig. S6 and Fig. S7), which was equivalent to 3.7×10^{10} copies/µl. Then, the prepared target DNA was serially diluted to concentrations of 3.7×10^{9} to 3.7×10^{-1} copies/µl.

Sensitivity of the ReCTC-based detection of *E. bieneusi*

The limit of detection (LOD) of the ReCTC was evaluated using the prepared target DNA with the aforementioned known concentrations. Fluorescence detection showed that samples with concentrations of 3.7×10^{0} copies/µl and higher target DNA showed strong fluorescence signals when recorded using the qTOWER^{3}G qPCR system and observed by the naked eye, compared with samples with lower concentrations and the negative control (Fig. 3a and b). Consistent results were also observed for the ReCTC-based LFS detection method. The lowest initial target DNA concentration that showed a test line was also 3.7×10^{0} copies/µl (Fig. 3c). These results indicated the LOD of the ReCTC-based nucleic acid detection of *E. bieneusi* was 3.7×10^{0} copies/µl of target DNA.

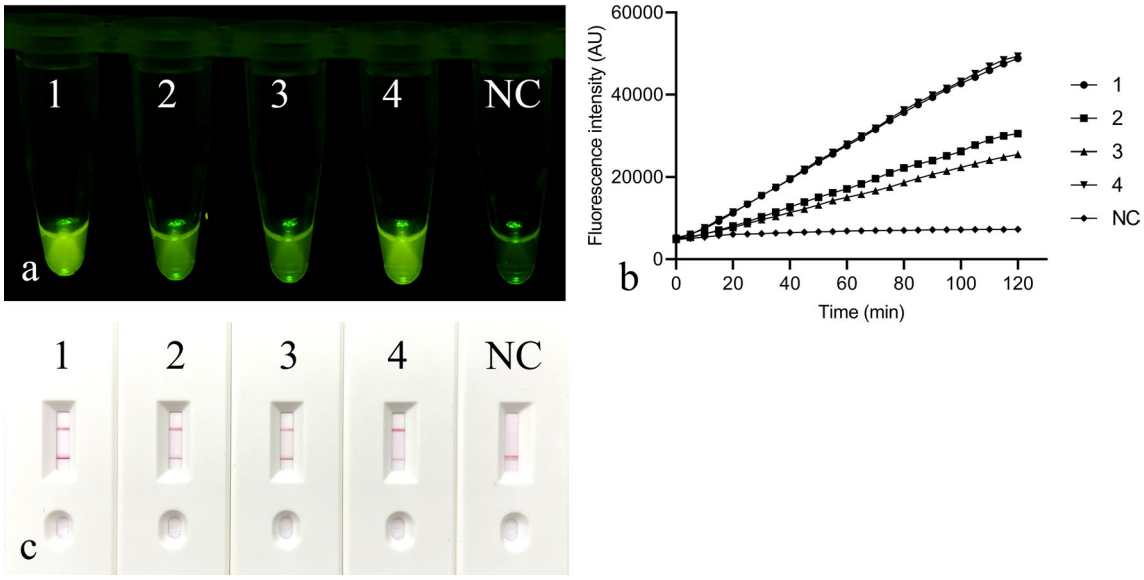

Fig. 2 Feasibility verification of the ReCTC-based detection. **a.** Obvious fluorescence signal can be visibly observed under UV light. **b**. Real-time fluorescence intensity curves of the ReCTC-based detection involving FAM-TTATT-BHQ1 reporter recorded using the qTOWER^{3}G qPCR system. **c**. Test lines in the ReCTC-based LFS detection assay involving FAM-TTATT-Biotin reporter. 1–4: Positive results; NC: negative result, ddH_2O as template

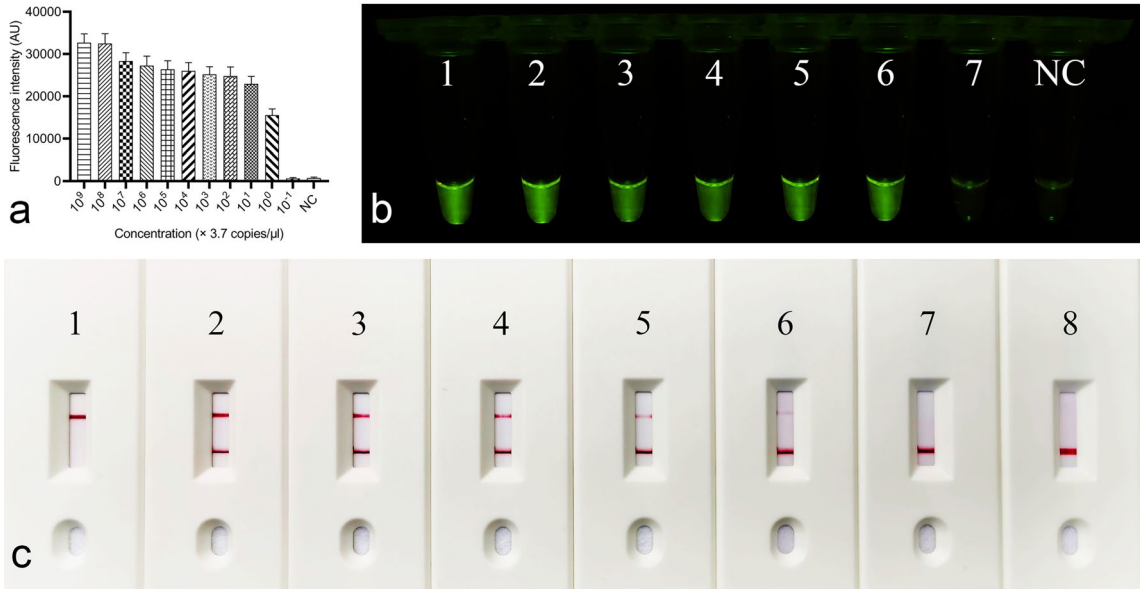

Fig. 3 Sensitivity of the ReCTC-based detection. **a**, **b.** Sensitivity test results of the ReCTC-based fluorescence assay using positive PCR products recorded using the qTOWER^{3}G qPCR system (**a**) and visibly observed under UV light (**b**). The LOD of the fluorescence assay was determined as 3.7×10^0 copies/μl of purified PCR products. **c.** Sensitivity test of the ReCTC-based LFS assay using positive PCR products. The LOD of the LFS assay was also determined as 3.7×10^0 copies/μl of purified PCR products. 1–7: The concentrations of purified PCR products were 3.7×10^5, 3.7×10^4, 3.7×10^3, 3.7×10^2, 3.7×10^1, 3.7×10^0 and 3.7×10^{-1} copies/μl, respectively. 8, NC: negative control, ddH_2O as template. According to the instructions on the strip product manual, only the detection line appears on the LFS when the concentration of target is very high. Because the LFS test reporter (FAM-TTATT-Biotin) were completely cut, then the FAM bound with colloidal gold nanoparticles flows forward and binds to the anti-FAM antibody at the detection line

Specificity of the ReCTC-based detection of *E. bieneusi*

The specificity of the ReCTC was verified using genomic DNA extracted from several prevalent intestinal protozoans in humans and animals, including *E. intestinalis*, *E. hellem*, *E. cuniculi*, *C. parvum*, *C. ryanae*, *Giardia duodenalis* and *Cyclospora cayetanesis*. When the initial DNA was positive for *E. bieneusi* the fluorescence intensity detected using the qTOWER^{3}G qPCR system was significantly higher than that of other protozoa, and strong fluorescence could also be visually observed under UV light (Fig. 4a and b). The specificity of the ReCTC-based nucleic acid detection of *E. bieneusi* was further

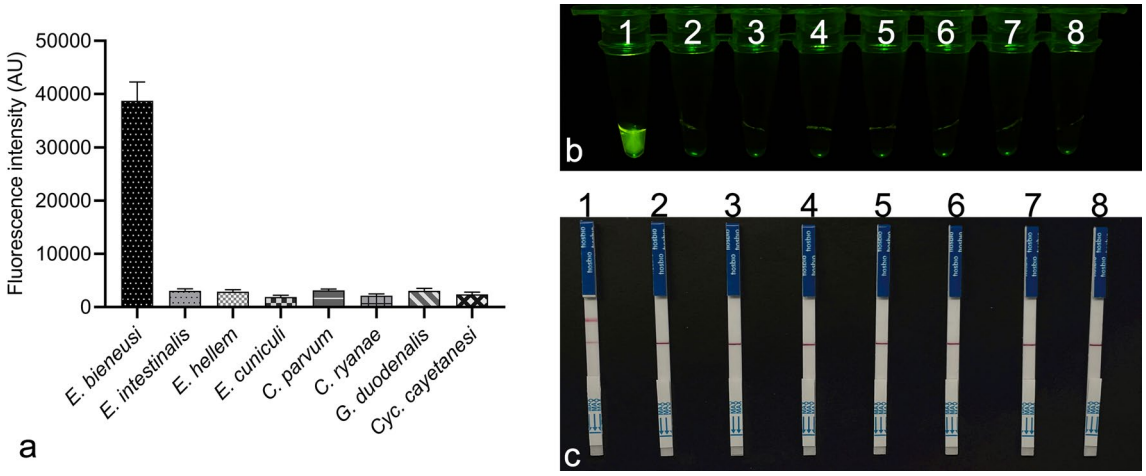

Fig. 4 Specificity of the ReCTC-based detection. Genomic DNA of *E. bieneusi*, *E. intestinalis*, *E. hellem*, *E. cuniculi*, *C. parvum*, *C. ryanae*, *Giardia duodenalis* and *Cyclospora cayetanesis* (1–8) were included. **a**, **b.** Specificity test results of ReCTC-based fluorescence assays recorded using the qTOWER^{3}G qPCR system (**a**) and visibly observed under UV light (**b**). Only samples of *E. bieneusi* exhibited strong fluorescence signals. **c**. Specificity test of the ReCTC-based LFS detection assay. A clear test line was observed only on the LFS that contained *E. bieneusi* DNA

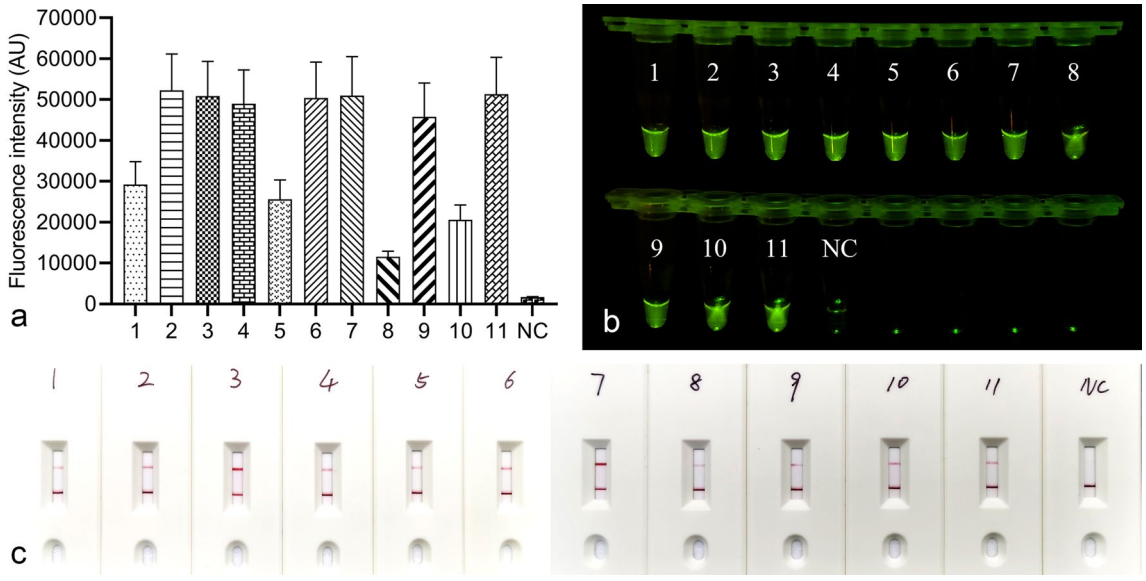

Fig. 5 ReCTC-based detection of *E. bieneusi* on clinical human samples. Known *E. bieneusi*-positive clinical fecal samples from 11 humans were tested using ReCTC-based fluorescence (**a**, **b**) and LFS (**c**) detection methods. All 11 samples showed positive results both in the ReCTC-based fluorescence and LFS detection methods. 1–11: Known *E. bieneusi*-positive clinical fecal samples from 11 humans; NC: negative control, ddH_2O as template

confirmed in the LFS assay, and a clear test line was observed only when *E. bieneusi* DNA was added (Fig. 4c).

Accuracy validation of the ReCTC-based detection of *E. bieneusi* in clinical samples

We validated the consistency between the ReCTC-based rapid assay and the most commonly used nested PCR assay for the detection of *E. bieneusi*. Eleven known *E. bieneusi*-positive samples, collected from hospitalized patients in Zhengzhou City and stored in our laboratory, were tested using both methods. Using nested PCR based on the ITS sequence, specific fragments of approximately 389 bp were successfully amplified from all samples, except for sample 8 (Additional File 1: Fig. S8). In the ReCTC-based fluorescence assay, all samples, including sample 8, showed fluorescence signals significantly higher than the negative control. The signals were recorded using the qTOWER^{3}G qPCR system and visually observed (Fig. 5a and b). All samples exhibited test lines on the strips in the ReCTC-based LFS assay (Fig. 5c). These results demonstrated that the method established here can successfully detect *E. bieneusi* in clinical samples. The sensitivity of the proposed method

was better than that of the nested PCR based on the ITS sequence.

Due to the vast variety in composition of various stool samples caused by diet, microbiome, and other factors, we tested 50 known *E. bieneusi*-negative samples collected from hospitalized patients in Zhengzhou City and stored in our laboratory stool samples to ensure that any unrelated DNA didn't give false positive. Using nested PCR based on the ITS sequence, no specific fragments of approximately 389 bp were successfully amplified from any of the samples (Fig. 6c and Additional File 1: Fig. S9). In the ReCTC-based fluorescence assay, no visible fluorescence was observed in 50 samples compared to positive control (Fig. 6a). Meanwhile, all samples only occurred control lines on the strips in the ReCTC-based LFS assay (Fig. 6b). The results of ReCTC-based detection method were consistent with the nested PCR based on the ITS sequence. These results demonstrated that the method established here can be used to detect *E. bieneusi* in clinical fecal samples without interference from unrelated DNA.

Discussion

E. bieneusi was first reported infesting the enterocytes of a Haitian patient with AIDS by Desportes et al. in 1985 [26] and has since been the subject of further study. However, understanding of the morphology and epidemiology of *E. bieneusi* has progressed slowly due to its inability to be pure cultured in vitro and the lack of effective detection and research methods [27, 28]. Microsporidiosis in humans is currently treated symptomatically. Although fumagillin and nitazoxanide are effective against infections caused by *E. bieneusi*, high toxicity and inaccessibility in most countries prevent their widespread use [11, 29]. In the absence of effective treatments, early detection of the infection caused by *E. bieneusi* is particularly important. Correct identification can lead to effective treatment and thus avoid empirical treatments of persistent diarrhea that are ineffective against *E. bieneusi*. Moreover, convenient and rapid testing can help control the further transmission of *E. bieneusi*.

Although methods exist for detecting *E. bieneusi* infections, they have trade-offs including sensitivity, specificity, simplicity, cost and speed [12]. The existing detection

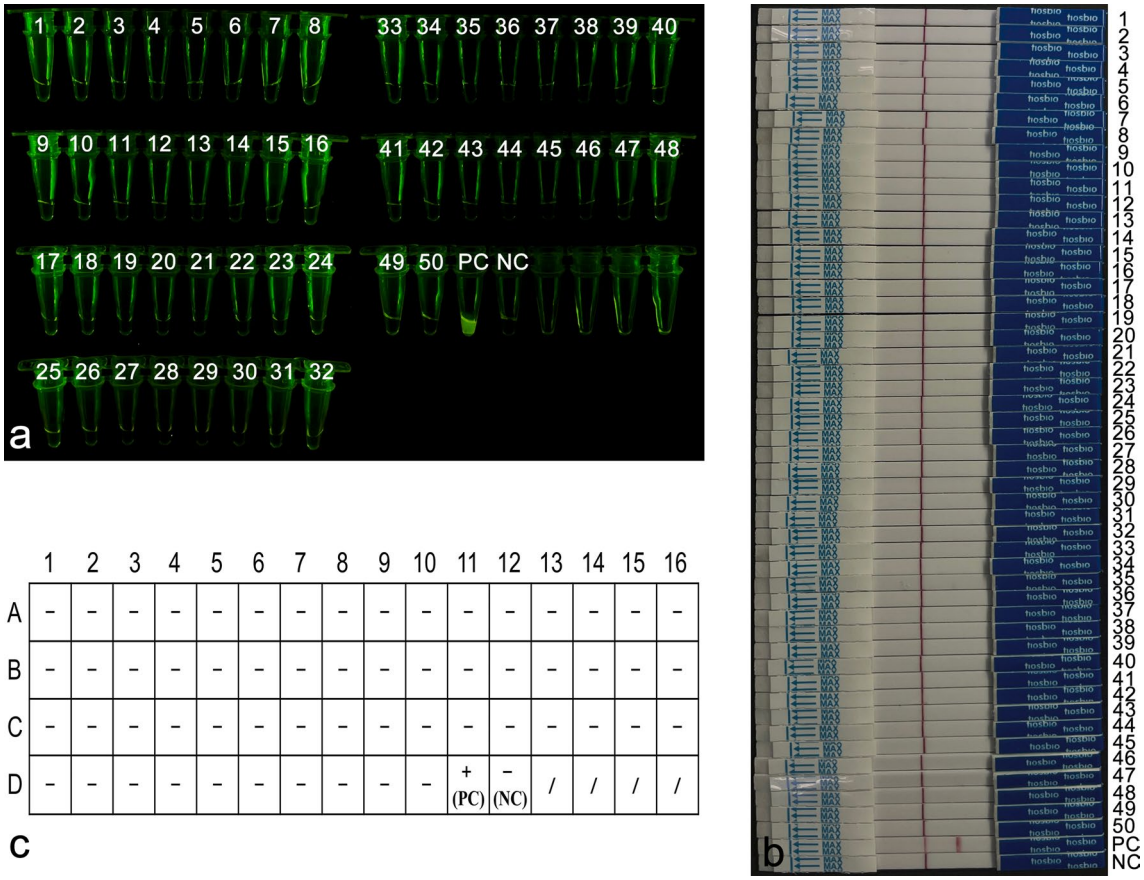

Fig. 6 ReCTC-based detection of *E. bieneusi* on clinical human samples. Known *E. bieneusi*-negative clinical fecal samples from 50 humans were tested using ReCTC-based fluorescence (**a**,) and LFS (**b**) detection methods. All 50 samples showed negative results both in the ReCTC-based fluorescence and LFS detection methods. 1–50: Known *E. bieneusi*-negative clinical fecal samples from 50 humans; PC: positive control; NC: negative control, ddH_2O as template

methods must be used in laboratories as they require special equipment and trained technicians. These detection methods are not feasible in resource-limited areas. The nonspecific *trans*-cleavage activity of Cas12a has been used for the rapid detection of pathogenic microorganisms, single nucleotide polymorphism detection, tumor screening and antibiotic resistance screening [30]. We previously reported a CRISPR/Cas12a-based diagnostic method, termed ReCTC, for the rapidly detection of *C. parvum* IId subtype family nucleic acid [23]. In the present study, we expanded the ReCTC and established an end-point nucleic acid detection method for *E. bieneusi* targeting the *swp* gene.

The small subunit rRNA gene is a commonly used locus in PCR detection of *E. bieneusi* because of its conservation. However, the PCR-based detection method can give false positive test results due to the cross-reactivity of the small subunit PCR primers with DNA from closely related microsporidia [24]. The ITS sequence is another fragment frequently used in the PCR detection of *E. bieneusi*. Unfortunately, when we screened the entire ITS sequence, no 20-nt to 24-nt fragment meeting the crRNA criteria was identified. The spore walls of microsporidia provide environmental protection and are also involved in host-pathogen interactions via species-specific spore wall proteins [24, 31, 32]. We selected a 20-nt fragment as the target sequence by multiple alignment of the *swp* gene and successfully applied it in ReCTC.

In this study, the potential target nucleic acid fragments (target DNA) in the samples were amplified in large quantities using the RPA assay. The amplification resulted in an increase exceeding 10^{12} [33]. Thus, RPA enabled signal amplification in the ReCTC and indicated high sensitivity. This was confirmed by a sensitivity test result, showing an LOD of 3.7 copies. The high sensitivity of the ReCTC might result from one activated Cas12a enzyme being able to cleave a large number of ssDNA probes in a short time once the amplicons were recognized and complementarily paired with the crRNA [18], This sent a strong fluorescence signal and resulted in a detection line on the test strip. Cas12a cleavage plays the role of signal conversion in the entire process, and it is also involved in signal amplification. This has been noted in previous reports [34].

The BLAST result showed that the sequences of other species, which had 100% identity and 78–86% sequence coverage, exhibited multiple base differences from the crRNA sequence. According to CRISPR/Cas12a detection system principle, the complementarity cannot tolerate two continuous nt mismatches [18]. Therefore, there is no false positive caused by cross-reaction. The high specificity of the ReCTC in the detection of *E. bieneusi* nucleic acid was confirmed in the specificity test involving other common enteric pathogens. The fluorescent signal and/or test line only appeared when the sample was positive for *E. bieneusi*. In the CRISPR/Cas system, the crRNA (or gRNA) sequences vary among different Cas enzymes and even among Cas12a orthologs. One of the conditions under which the enzyme is activated is restricted to the reverse complementarity between the spacer region of the crRNA (or gRNA) and the protospacer of the target sequence; the complementarity can tolerate only one or two discontinuous nt mismatches [18]. This indicates that the DNA fragment to be tested must have nearly 100% homology with crRNA, and this is one of the prerequisites for the specificity of the ReCTC. The RPA primers also have high specificity, and this contributes to the specificity of the ReCTC. Only a DNA fragment that is homologous with RPA primers can be successfully amplified, and only the amplified target DNA that is homologous with crRNA can cleave the ssDNA probe. Therefore, the fluorescence signal visually observed or the test line shown on the LFS is only seen if there are orthologs present in the test samples.

As an end-point detection method, when the ReCTC is used in clinical DNA samples, the results can be obtained in approximately 1 h. This is approximately half of the time required by the nested PCR method [14]. This method is more convenient and provides interpretations that are independent of professional technicians or sophisticated equipment. The ReCTC also has advantages compared with other detection methods. We compared the detection efficiency of the ReCTC and the nested PCR in clinical *E. bieneusi*-positive human DNA samples and found that false negatives appeared in the PCR method but not in the ReCTC method. The amount of *E. bieneusi* in fecal samples was low, resulting in the target DNA in extracted DNA solution being lower than the minimum detection concentration of nested PCR method, which resulted in the sample 8 failed in the nested PCR method. These results also confirmed the advantage of the ReCTC method when used for point-of-care (POC) detection.

There are limitations in the ReCTC-based rapid detection method of *E. bieneusi* established in this study. First, quantitative detection was not achieved due to the nonlinear dependence in both the signal conversion process and the RPA amplification [35]. Second, the method requires RPA amplification and then the addition of amplicons into the CRISPR/Cas12a detection system. This requires two sample addition operations and increases the risk of cross-contamination. Integration of the two reactions in a reaction vessel to create a "one-pot" assay would be an improvement.

Conclusion

The previously established ReCTC was used for the detection of *E. bieneusi* nucleic acid with high sensitivity and specificity. The method was applied to clinical DNA samples and performed better than the nested PCR method. This new method shows broad application potential in POC detection, especially in resource-scarce areas. It could play a role in the prevention and control of *E. bieneusi* in humans and animals.

Abbreviations

Cas	CRISPR-associated
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
dsDNA	Double-stranded DNA
ITS	The internal transcription spacer
LFS	Lateral flow strip
LOD	Limit of detection
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
POC	Point of care
ReCTC	Recombinase polymerase amplification and Cas12a/crRNA trans-cleavage
RPA	Recombinase polymerase amplification
SNP	Single nucleotide polymorphism
ssDNA	Single-stranded DN A
SSU rRNA	Small subunit ribosomal RNA
SWP	Spore wall protein
nt	Nucleotide
TEM	Transmission electron microscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04391-3.

Supplementary Material 1

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Author contributions

YLW: Conceptualization; Data curation; Writing - original draft. FCY: Conceptualization; Data curation; Writing - original draft. KHZ: Formal analysis; Validation. KS: Validation; Grant funding. YCC: Software. JQL: Supervision; Writing - review & editing. XYL: Supervision; Writing - Review & Editing. LXZ: Conceptualization; Supervision; Grant funding; Writing - Review & Editing.

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Data availability

The data supporting this study's findings are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate

All the research procedures used in this study were approved by the Institutional Review Board of Henan Agricultural University (approval no. IRB-HENAU-20190820-02). The use of positive human samples in this study complied with the 1975 Declaration of Helsinki, as revised in 2013.

Consent for publication

No applicable.

Competing interests

The authors declare no competing interests.

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